Parallel FoxP1 and FoxP2 Expression in Songbird and Human Brain Predicts Functional Interaction

Ikuko Teramitsu, Lili C. Kudo, Sarah E. London, Daniel H. Geschwind, and Stephanie A. White

Introduction

The neural mechanisms for auditory-guided vocal learning are incompletely understood. Surprisingly, vocal learning (i.e., the ability to substantially modify innate vocalizations to mimic a vocal model) is evident in only a few animal groups, including songbirds and humans but not other primates or rodents (Snowdon and Hausberger, 1997; Doupe and Kuhl, 1999). In songbirds, the development and production of learned song is subserved by interconnected regions of the pallium (also called cortical mantle), striatum, and thalamus, collectively known as the song circuit (Fig. 1) (Bottjer and Johnson, 1997; Farries, 2001). Identification of the molecules that define and operate within this circuit would provide insight into the neural mechanisms for song learning, enable comparison to humans, and possibly reveal shared mechanisms for vocal learning. For example, a molecule known as synelfin in songbirds and α-synuclein in mammals is regulated in song circuitry during song learning and has been linked to Parkinson’s and Alzheimer’s diseases in humans (Clayton and George, 1999). Such findings indicate that common mechanisms may underlie specific motor and memory processes in birds and humans.

Recently, FOXP2, which encodes a member of the Forkhead box (Fox) family of proteins, has been identified as the gene underlying a human developmental language abnormality (Lai et al., 2001). FOX proteins are transcriptional regulators characterized structurally by a DNA-binding domain that forms a winged helix and functionally as embryonic morphogenerators (Carlsen and Mahlapuu, 2002) [See Kaestner et al. (2000) and http://www.biology.pomona.edu/fox.html for nomenclature. Briefly, nucleotide sequences are italicized whereas proteins are not. Human forms are capitalized (e.g. FOXP2 protein), murine forms are in lowercase (e.g. Foxp2), and those of other species, such as the zebra finch, are in uppercase and lowercase (e.g. FoxP2).] The FOXP subfamily has four members and is distinguished by a divergent winged-helix domain and a novel zinc finger motif (Shu et al., 2001; Lu et al., 2002). Whereas Foxp3 is expressed in T-cells (O’Garra and Vieira, 2003), Foxp1, Foxp2, and Foxp4 are implicated in lung development. They are also expressed in brain (Shu et al., 2001; Lu et al., 2002; Ferland et al., 2003; Takahashi et al., 2003). However, no neural role had been hypothesized for these molecules before discovery of the human mutation in FOXP2.

Individuals with a FOXP2 mutation exhibit prominent deficits in orofacial movements, called buccal-oral apraxia, but perform normally for simple oral and limb movements (Vargha-Khadem et al., 1998; Alcock et al., 2000a; Watkins et al., 2002a). They are impaired on tests of verbal fluency and language com-
prehension, in addition to language production (Marcus and Fisher, 2003). These behavioral features are accompanied by structural abnormalities in the cortex and striatum among other brain regions, and atypical activity of a corticostriatal network that participates in both covert and overt speech (Lai et al., 2001; Belton et al., 2003; Liegeois et al., 2003). Although the affected phenotype is not limited to language, linguistic difficulties are prominent (Vargha-Khadem et al., 1995), indicating that FOXP2 acts as a core deficit in complex coordination of genes that are also affected by FoxP2, and can dimerize with FoxP2, shares similar domains whereby it represses transcription of overlying expression in some song nuclei. FoxP1 exhibits a more striking sexual dimorphism, nearly concordant with the sexual dimorphism of the song circuit. These discoveries in zebra finch brain motivated us to examine FOXP1 and FOXP2 in human brain, in which expression was again found to partially overlap in striatal and thalamic structures. This unique comparative approach between songbird and human implicates both genes in the formation and function of circuitry that uses sensory feedback to learn voluntary, sequential, orofacial gestures.

Materials and Methods

**Animals and tissues.** All animal use was approved by the University of California, Los Angeles Institutional Animal Care and Use Committee.

**Human tissues.** Human tissue was obtained from the Brain and Tissue Bank for Developmental Disorders at the University of Maryland (Baltimore, MD). Gestational age was estimated based on the mother’s last menstrual period. After fetal extraction, the tissue was frozen rapidly on dry ice and stored at −80°C before sectioning. Postmortem intervals ranged between 0.1 and 3 hr. Tissue from four fetal brains was used. Human fetal stages were chosen to correspond with the time when neurogenesis for subcortical structures, such as the basal ganglia and thalamus, is mostly complete, when the tissue quality for RNA studies is high enough to be comparable to experimental animals (short postmortem interval), and when the brain is small enough to be assessed in a single large slice.

Isolation of zebra finch FoxP1 and FoxP2 cDNAs. Oligo(dT)-primed total RNA was reverse transcribed to cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Partial cDNAs were first amplified by PCR using degenerate primers designed to hybridize with the zinc finger and Fox domains in mouse and human FoxP1 and FoxP2 (sense 5'-MGRGTDCAAATGCARGTKGT-3'; antisense 5'-TGMGBCAGTGCRTTCTCCA-3'). A FoxP1 cDNA fragment 3' to the obtained segment was then isolated from the heart and brain using a sense primary specific to zebra finch FoxP1 (5'-CGTGGTTACCGAGTGTTCG-3') and a degenerate antisense primer (5'-CATTCTAGTCGTTCTACGG-3'). This 3' FoxP1 coding fragment revealed the zebra finch-specific sequence that was then used to obtain the first probe for in situ hybridization (see below). The entire ORF of FoxP2 was subsequently obtained from brain cDNA reverse transcribed from poly(A) RNA using the Marathon cDNA amplification kit (Becton Dickinson Biosciences, San Jose, CA) with primers based on consensus sequences within mouse (accession number AY079003) and human (accession number AF337817) FoxP2 5' and 3' untranslated regions (UTRs) (sense 5'-AGAGAAAGGTATTAAGTC-3'; antisense 5'-GCTTGAATTATCCTTTAGG-3'). PCR cycling conditions using the Advantage cDNA PCR kit (Becton Dickinson Biosciences) were: (1) FoxP1/FoxP2 fragments with consensus degenerate primers (2 min at 94°C for 1 cycle; 15 sec at 94°C, 30 sec at 58°C, and 1 min at 72°C for 35 cycles; and 30 sec at 72°C for 1 cycle); (2) FoxP1/FoxP2 fragments with degenerate primers (2 min at 94°C for 1 cycle; 15 sec at 94°C, 30 sec at 58°C, and 1 min at 72°C for 35 cycles; and 30 sec at 72°C for 1 cycle); (3) full-length FoxP2 (2 min at 94°C for 1 cycle; 15 sec at 94°C, 30 sec at 46°C, 4 min at 72°C for 35 cycles; and 3 min at 72°C for 1 cycle). Amplified cDNAs were cloned into pCR 4-TOPO vector (Invitrogen) and sequenced in sense and antisense directions. For the full-length FoxP2 cDNA, a total of 15 independent subclones were sequenced. For the two FoxP1 fragments, two subclones were sequenced for the first fragment and 10 subclones for the second, more 3' fragment.

Zebra finch probe synthesis. Two distinct regions each from FoxP1 and FoxP2 were chosen for generating probes for in situ hybridization analyses to ensure the specificity of the expression patterns observed for each gene. The first probes were designed to hybridize to the 3' portion of FoxP1 and FoxP2, respectively. For FoxP1, this was the region corresponding to 1708–2011 bp of human FOXP1 (accession number NM_032682) relative to the start codon. For FoxP2, this was 1870–2127 bp of...
the newly cloned zebra finch FoxP2 relative to the start codon. cDNA fragments coding these regions were amplified by PCR with primers designed from zebra finch FoxP1 and FoxP2 sequences, respectively (FoxP1: sense 5′-AATGCTGGTTTACAGGCT-3′, antisense 5′-GGTCATCTTCATATCTC-3′; FoxP2: sense 5′-ATAAAATACCGCATGTTGGCG-3′, antisense 5′-TTTCCAGATCTTCAGATAAAGGCC-3′). Cycling conditions were as mentioned above.

The second probes were designed to hybridize to the coding region upstream of the zinc finger domain of each of the genes (FoxP1: region corresponding to 661–998 bp of human FOXP1, accession number NM_032682; FoxP2: 676–1005 bp of zebra finch FoxP2, accession number AY397909). Of note, if the zebra finch possesses variant FoxP transcripts, as observed in the mouse (Shui et al., 2001), this second probe should recognize multiple forms. FoxP1 and FoxP2 cDNA fragments coding these second probe regions were amplified by PCR using the Advantage 2 PCR kit (Becton Dickinson Biosciences) with primers designed from chicken FoxP1 (accession number BQ 038849) and zebra finch FoxP2 sequences, respectively (FoxP1: sense 5′-CAAGGCATTGACAGAAACAGTC-3′, antisense 5′-AGCGGATCTCATGTTTAGATG-3′; FoxP2: sense 5′-CATCTGCTGG-3′; FoxP2: antisense 5′-AGTATGGGAGGGCCGCGTCT-3′). Cycling conditions for both primer sets were: (1) 1 min at 95 °C for 1 cycle; (2) 20 sec at 95°C, 1 min at 68°C for 35 cycles; (3) 1 min at 68°C for 1 cycle; and (4) 10 min at 70°C for 1 cycle. Amplified fragments were subcloned into pcCR4-TOPO vector (Invitrogen), sequenced to confirm their identity, and then used for in vitro transcription to generate sense and antisense RNA probes labeled with [33P]UTP (Amersham Biosciences, Piscataway, NJ) using Riboprobe Combination System-T3/T7 (Promega, Madison, WI).

Human probe synthesis. cDNA from the left temporal cortex of a 19 week human fetal brain was obtained as follows: total RNA was extracted from the 5–7 mm thick coronal and sagittal sections were thaw-mounted onto 50 μm thick coronal and sagittal sections were thaw-mounted onto 50 μm thick sections; (3) similar expression patterns occurred across multiple ages; (4) signals from film and emulsion-dipped sections corresponded; and (5) signals were detected with the antisense, but not with the sense probes.

In situ hybridization analyses of zebra finch. Analysis of FoxP gene expression was performed essentially as described by Jacobs et al. (1999), except that frozen sections were thaw-mounted on Super Frost Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and postfixed with 4% paraformaldehyde, pH 7.4. Briefly, a series of 20 μm thick coronal or sagittal sections were hybridized with [33P]UTP-labeled RNA probes. Five sets of slides containing adjacent sections were used, a set each for FoxP1 sense, antisense and FoxP2 sense, antisense probes. Equivalent counts per minute of sense and antisense probes for both FoxP1 and FoxP2 were loaded per slide. The fifth set was stained with thionin (Tolivia and Tovilla, 1985) to enable identification of neuroanatomical structures and to guide localization of the expression patterns for each gene with reference to a songbird brain atlas (Stokes et al., 1974) (Table 1). Wherever possible, specific structures were named, but when the anatomy was less clear, as in d1 bird brains, more general descriptions were used. After hybridization, slides were apposed to autoradiographic film (BioMax MR film; Eastman Kodak, Rochester, NY) for 24–48 hr or 48–72 hr for FoxP1 or FoxP2, respectively. Slides were then dipped in liquid emulsion (NTB-2; Eastman Kodak) and exposed at 4°C for 4 or 5 weeks, for FoxP1 or FoxP2, respectively. Emulsion-coated slides were developed, dehydrated, and coverslipped for determination of expression patterns.

Several criteria were applied to assign the observed radioactive signals to specific neuroanatomical regions (the latter were identified by Nissl stains and by reference to an atlas, as mentioned above). For each anatomi- cal designation: (1) signals were detected by each of two non-overlapping probes for a given gene; (2) signals were observed in consecutive sections; (3) similar expression patterns occurred across multiple birds (n ≥ 3 per age); (4) signals from film and emulsion-dipped sections corresponded; and (5) signals were detected with the antisense, but not with the sense probes.

In situ hybridization analyses of human brains. In situ hybridization analyses of FOXP genes in human brain tissue were performed essentially as described by Geschwind et al. (2001) for human tissues, except that 20 μm thick coronal and sagittal sections were thaw-mounted onto 50 μm thick sections for 50 × 70 mm slides (Brain Research Laboratories, Newton, MA). These were dried and postfixed in 4% buffered paraformaldehyde, pH 7.4, for 20 min at room temperature, rinsed in 0.1 M phosphate buffer and water, and air dried for 30 min. Sections were stored desiccated at −80 °C before use. Briefly, before hybridization, slides were treated with glycine and acetic anhydride and TEA, followed by two 2× SSC washes and a series of ethanol washes. Hybridized slides were incubated overnight at 60°C. The slides were then washed twice in 4× SSC at 60°C, treated with RNase A in 45°C, washed four times in 2× SSC at room temperature, twice in 0.5×

<table>
<thead>
<tr>
<th>Table 1. Neuroanatomical abbreviations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Abbreviation</strong></td>
</tr>
<tr>
<td><strong>Songbird terminology</strong></td>
</tr>
<tr>
<td>Area X</td>
</tr>
<tr>
<td>DLM</td>
</tr>
<tr>
<td>DTZ</td>
</tr>
<tr>
<td>GP</td>
</tr>
<tr>
<td>HA</td>
</tr>
<tr>
<td>HD</td>
</tr>
<tr>
<td>LMAN</td>
</tr>
<tr>
<td>Meso</td>
</tr>
<tr>
<td>MLd</td>
</tr>
<tr>
<td>N</td>
</tr>
<tr>
<td>Ov</td>
</tr>
<tr>
<td>RA</td>
</tr>
<tr>
<td>RPGc</td>
</tr>
<tr>
<td>Rt</td>
</tr>
<tr>
<td>SN</td>
</tr>
<tr>
<td>SML</td>
</tr>
<tr>
<td>SMI</td>
</tr>
<tr>
<td>SPC</td>
</tr>
<tr>
<td>Spl</td>
</tr>
<tr>
<td>TeO</td>
</tr>
<tr>
<td>VTA</td>
</tr>
<tr>
<td><strong>Human terminology</strong></td>
</tr>
<tr>
<td>Adl</td>
</tr>
<tr>
<td>Avl</td>
</tr>
<tr>
<td>Caud</td>
</tr>
<tr>
<td>CM</td>
</tr>
<tr>
<td>CP</td>
</tr>
<tr>
<td>GPi</td>
</tr>
<tr>
<td>IZ</td>
</tr>
<tr>
<td>MD</td>
</tr>
<tr>
<td>MZ</td>
</tr>
<tr>
<td>P</td>
</tr>
<tr>
<td>Pcn</td>
</tr>
<tr>
<td>PF</td>
</tr>
<tr>
<td>Ro</td>
</tr>
<tr>
<td>SP</td>
</tr>
<tr>
<td>Str</td>
</tr>
<tr>
<td>VA</td>
</tr>
<tr>
<td>VB(VPL/VPM)</td>
</tr>
<tr>
<td>VL</td>
</tr>
<tr>
<td>Vlc</td>
</tr>
<tr>
<td>VM</td>
</tr>
</tbody>
</table>
SSC at 60°C, once in 0.1× SSC at 60°C, once in 0.1× SSC at room temperature, then rinsed in water. After hybridization, slides were apposed to autoradiographic film (BioMax MR film; Eastman Kodak) for a period of 5 d, after which the film was developed under standard conditions. Control sections incubated with sense RNA showed no specific hybridization. Slides were then defatted in a series of ethanol and chloroform, dipped in NTB-2 emulsion (Eastman Kodak), and stored at 4°C for 4–5 weeks. Slides were developed in D-19 developer (Eastman Kodak) and fixed in Kodak Fixer both at 15°C. After development, slides were stained with cresyl violet, followed by a series of ethanols, left in Citrisolv (Fisher Scientific) overnight, then coverslipped with Permount (Fisher Scientific).

Analysis of human brain structures labeled with cRNA probes was performed on both autoradiograms and emulsion-dipped slides stained with cresyl violet for optimal anatomical resolution. The use of a combination filtered bright field with dark-field epi-illumination (Darklite; Nikon, Melville, NY) allowed for simultaneous visualization of silver grains and cresyl violet-stained cells, facilitating structural analyses. Brain structures, including specific thalamic nuclei, were identified and labeled according to two reference atlases for primate embryonic brain and thalamus (Olszewski, 1952; Jones, 1985; Feess-Higgins and Larroche, 1987), with additional discussion with Dr. Todd Preuss (Emory University, Atlanta, GA), an expert primate comparative anatomist.

**Results**

**FoxP2 sequence in an avian vocal learner**

Among the identified primate FoxP2 sequences, human FOXP2 bears unique residues at positions 303 and 325 (Fig. 2). During evolution, these substitutions are postulated to be key molecular events that gave rise to language, or minimally to the capacity for selection and sequencing of orofacial movements required for speech (Enard et al., 2002; Zhang et al., 2002; Clark et al., 2003). To discover whether songbirds, being vocal learners, possess the human substitutions, we cloned the full-length FoxP2 sequence from the zebra finch using primers designed based on consensus sequences of the mouse and human 5’ and 3’ UTRs. The complete cDNA encodes a predicted protein of 709 amino acids that contains a poly-glutamine tract (aa 152–225; data not shown), as well as the putative zinc finger domain (aa 336–366) and the Fox domain (aa 489–579), which are characteristic features of the FoxP subfamily (Shu et al., 2001). Regions of the zebra finch deduced amino acid sequence are aligned with those from human, chimpanzee, and mouse in Figure 2. The zebra finch sequence demonstrates 97% and 100% identities with the human homolog in the zinc finger and Fox domains, respectively. Of note, all amino acids shared between the two vocal learners, zebra finch and human, are shared with nonhuman primates as well. Because the zebra finch does not possess the human-specific residues, yet can modify its vocalizations, it appears that no single FoxP2 sequence accounts for all instances of this behavioral trait. Intriguingly, zebra finch FoxP2 possesses five residues that are distinct from residues at the corresponding position in all mammalian homologs identified thus far (Fig. 2).

**FoxP2 in both sensory and motor structures of adult male zebra finch brain**

The structural and functional deficits observed in the cortex and striatum of human beings bearing a FOXP2 mutation suggested that FOXP2 expression would be localized to these regions (Vargha-Khadem et al., 1998; Liegeois et al., 2003; Watkins et al., 2002b; Fisher et al., 2003). In songbirds, a cortico-striato-thalamo-cortical loop underlies the development and production of learned song (Fig. 1A) (for review, see Bottjer and Johnson, 1997; Farries, 2001). To determine whether the song circuit expresses FoxP2, we performed in situ hybridization on brain sections from adult male zebra finches (>d150) whose neural structures are fully developed. In the telencephalon, FoxP2 is expressed at low levels in pallial (cortical) regions and high levels in the striatum, as hypothesized based on the structural and functional abnormalities of afflicted humans. There is substantial diencephalic expression and specific expression in some mesencephalic structures (Fig. 3–5). Interestingly, in addition to motor structures, FoxP2 is expressed in visual and auditory processing regions consistent with a potential role in sensory feedback.

The recent renaming of the avian brain facilitates meaningful comparisons with other vertebrates and indicates that pallial regions of the avian telencephalon are broadly homologous to the mammalian neocortex, claustrum, and pallial amygdala (Reiner et al., 2004). Within adult zebra finch telencephalon, FoxP2 is broadly expressed at low levels in the hyperpallium densocellular (HD) and mesopallium and at even lower levels in the nidopallium (Fig. 3A). The specificity of this label is indicated by the comparative lack of signal in the arcopallium (Fig. 4A), in field I of the nidopallium (Fig. 5D), and, by comparison, with a sense control from an adjacent section (Fig. 3A). The song nucleus HVC (used as a proper name) is labeled, but not above the level of the surrounding nidopallium (Fig. 5A), as confirmed by visual inspection of emulsion-dipped slides (data not shown). The song nucleus lateral magnocellular nucleus of the anterior nidopallium (LMAN) shows signals at or below the level of the surrounding nidopallium (Figs. 3A, 5D). As mentioned above, the arcopallium, including the premotor song nucleus, robustus arcopallialis (RA), is not labeled (Figs. 4A, 5B, C).

In contrast to the limited pallial expression, strong FoxP2 signal is detected in the avian striatum, striatum mediale (StM), and striatum laterale. Within the StM, the specific area required for song development, called area X (Sohrabji et al., 1990; Scharff and Nottebohm, 1991), expresses FoxP2 at a level comparable with or
slightly higher than the surrounding regions (Fig. 3B, 5D). This strong striatal expression is consistent with the structural abnormalities observed in the caudate nucleus in affected humans (Watkins et al., 2002b; Belton et al., 2003). The globus pallidus (GP) is a major telencephalic component that exhibits sparsely distributed FoxP2 signals in the finch brain (Figs. 3C, 4B, 5C). It is worth noting that area X contains a pallidal component intermingled with the striatal one, and together these are proposed to comprise a pathway equivalent to the direct striato-pallido-thalamic pathway, mediated by globus pallidus interna (GPI) in the mammalian basal ganglia (Farries and Perkel, 2002). Therefore, FoxP2 signals within area X could include pallidal expression; however, our methods did not allow us to discriminate this. Congruent with this idea, human fetal GPi expresses FoxP2 (see below).

In the diencephalon, both dorsal and ventral thalamic structures strongly express FoxP2. The dorsal thalamic zone (DTZ) (Veenman et al., 1997), located dorsomedially in the avian diencephalon, shows distinct subregional labeling (Figs. 3C, D, 5A). The DTZ is homologous to the mammalian intralaminar, midline, and mediodorsal thalamic nuclear complex (IMMC) (Veenman et al., 1997). It consists of multiple nuclei with boundaries that likely underlie the observed pattern of FoxP2 expression. For example, nucleus dorsolateralis anterior thalami, pars medialis (DLM), part of the song circuit, expresses FoxP2 mRNA, as does dorsomedial thalamus, whereas nucleus dorsolateralis anterior thalami, pars lateralis does not (Fig. 3C, D). Detailed immunohistochemical methods coupled with anterograde and retrograde tracing will be required for a more specific designation. In the vicinity of, but histologically distinct from, the DTZ is the ventrointermediate area (VIA), a region described in pigeons as comparable to the motor part of the mammalian ventral tier (Medina et al., 1997). In the zebra finch, FoxP2 signals are visible in this region just medial to the nucleus rotundus (Rt) (Fig. 3C).

A dorsal structure involved in visual processing, nucleus superficiale parvocellularis (Fig. 3D) (Trottier et al., 1995), is labeled. Other sensory thalamic nuclei with strong expression include ovoidalis (Ov), a major auditory input (Brauth and Reiner, 1991; Knudsen et al., 1993; Bruce et al., 2002), and Rt, which receives visual input from the tectum opticum (TeO) (Figs. 3C, D, 4D).

Sensory midbrain regions with substantial levels of FoxP2 include the auditory nucleus, mesencephalicus lateralis, pars dorsalis (MLd) (Fig. 5B), and TeO (Figs. 3D, 5A, B). Expression in mesencephalic motor regions includes label within the substantia nigra (Fig. 5B) and distributed label in the region containing the nucleus ruber (data not shown) (Wild et al., 1979). FoxP2 is expressed in the ventral tegmental area (Fig. 5A), which sends dopaminergic projections into area X (Lewis et al., 1981) and receives projections from the nucleus of the basal optic root. These nuclei are part of the accessory optic system in vertebrates, involved in multisensory analysis of self-motion (Wylie et al., 1999). In metencephalon, strong FoxP2 signals are observed in the vicinity of the nucleus reticularis pontis caudalis, pars gigantocellularis (Fig. 5B). These neurons are thought to play a role in the acoustic startle response and the sensorimotor integration of head-orienting movements (Nodal and Lopez, 2003; Park et al., 2003; Sasaki et al., 2004). In the cerebellum, Purkinje cells express FoxP2 (Fig. 5B, D). All FoxP2 signals were distributed symmetrically across hemispheres, as expected, given that lateralization of vocal control structures in songbirds is primarily peripheral (Suthers, 1997). Signals obtained with the second FoxP2 probe were identical to those obtained with the first (Fig. 4D) (see Materials and Methods).
**FoxP1 is expressed in song nuclei**

*FoxP1* was investigated in addition to *FoxP2*, because it can dimerize with FoxP subfamily members (Wang et al., 2003; Li et al., 2004), shares a similar repressor domain, and can repress transcription from the same lung-specific promoters (Shu et al., 2001). Strikingly, *FoxP1* shows expression within song circuit structures that are sexually dimorphic in zebra finches. In nidopallium, the song nucleus HVC shows enhanced expression (Figs. 3C, 4C, 5A,C). Within the arcopallium, *FoxP1* is clearly expressed in the song nucleus RA (Figs. 4A, 5B,C) and slightly expressed ventrolateral to RA in the arcopallium dorsale (Ad) (Fig. 5B). Of the remaining pallium, *FoxP1* is strongly expressed in HD and the mesopallium (Figs. 3A, B, 5C,D). Within the nidopallium, *FoxP1* expression appears stronger rostral versus caudal, to field L (Fig. 5D). There is a striking lack of signal in the vicinity of the nucleus basorostralis pallii (Bas) (Fig. 3A, B, arrowheads).

As in mouse lung (Shu et al., 2001; Lu et al., 2002), *FoxP1* expression in zebra finch striatum and thalamus partially overlaps with that of *FoxP2*. Intriguingly, within the StM, area X is strongly labeled, above the level of the surrounding striatum (Figs. 3B, 5D). *FoxP1* expression in area X is more evident than that of *FoxP2*, indicating that the stronger *FoxP1* signal is not simply attributable to increased cell density but rather reflects enhanced expression in area X. The GP appears to lack *FoxP1* expression (Fig. 4B). Within the DTZ, *FoxP1* expression overlaps with that of *FoxP2*, including within the DLM, the thalamic component of the song circuit (Fig. 3C,D). Unlike *FoxP2*, *FoxP1* was not expressed in the sensory input nuclei Ov or Rt (Fig. 3C, D). Whereas human brain imaging did not resolve specific structural thalamic deficits related to the human FOXP2 mutation, our zebra finch data indicate that the thalamus, in addition to the striatum, is another key site of *FoxP1* and *FoxP2* expression.

Mesencephalic *FoxP1* signals, like those of *FoxP2*, occur in the aforementioned sensory regions MLD (Fig. 5B) and TeO (Figs. 3D, 4E, 5A,B), although at lower levels. A visual processing nucleus, spiriformis lateralis (SpL) (Toledo et al., 2002), strongly expresses *FoxP1* (Fig. 4E). As with *FoxP2*, all *FoxP1* signals were observed bilaterally, and patterns observed with the second probe were identical to those obtained with the first (Fig. 4E) (see Materials and Methods). In summary, both *FoxP* genes are expressed in visual and auditory nuclei critical for the sensory feedback required for song learning (Konishi, 1965; Morrison and Nottebohm, 1993), in addition to motor control regions.

**FoxP1 and FoxP2 in developing zebra finch brain**

As with many other Forkhead (Fox) transcription factors, Foxp1, Foxp2, and Foxp4 are implicated in organogenesis, specifically in lung and heart development (Shu et al., 2001; Lu et al., 2002). Recent studies of the KE family indicate that Foxp2 is critical for brain development (Lai et al., 2001) because its mutation leads to specific structural (Watkins et al., 2002b) and functional (Liegeois et al., 2003) neural deficits while apparently sparing the lungs and heart (Marcus and Fisher, 2003). We, therefore, examined both *FoxP1* and *FoxP2* expression in the developing brain of male zebra finches at 1 d after hatching (d1) and during the song-learning period at d35. At d1, the gross expression patterns of...
FoxP1 and FoxP2 resemble those in adults (Fig. 6A). Substantial FoxP1 mRNA is detected in regions that correspond to the pallium and striatum of adult telencephalon. FoxP2 is highly expressed in the striatum. Interestingly, strong signals are observed in regions lying near, but not directly adjacent to, the ventricle (data not shown), indicating a potential role for FoxP2 during migration or differentiation of neurons. In d35 brains, the specific expression patterns of FoxP1 and FoxP2 observed in adults are already evident, including strong expression of FoxP1 in the song nuclei, area X (Fig. 6B), HVC (Fig. 6F), and RA (data not shown) and subregional expression within the DTZ of both genes (Fig. 6C).

Sites of sexual dimorphism
Given the expression of FoxP1 and FoxP2 in many song nuclei and in sensory and motor pathways that are also crucial for malespecific song learning, we asked whether such expression patterns are unique to male zebra finches. Our results reveal that FoxP2 expression does not differ consistently between sexes at juvenile or adult ages. For example, in d35 females, FoxP2 mRNA is prominent in the striatum and the aforementioned regions of the dorsothalamus (Fig. 6D, E). In some sections of d35 and adult male brains (Fig. 5D), the outline of area X is faintly discernible to the naked eye. However, this visibility was inconsistent between individual birds in both age groups. In sharp contrast, FoxP1 expression at d35 shows a consistent sexual dimorphism, corroborating the sexual dimorphism of the song circuit. In particular, in females, no enhancement of FoxP1 expression above the level of the surrounding striatum is detected in the StM, where the song nucleus, area X, is present in males (Fig. 6D, compare B, D). The FoxP1 riboprobe, however, detects mRNAs in the smaller RA of females as well as in adjacent Ad (Fig. 6G). More detailed investigation will determine whether any enhanced FoxP1 expression occurs in the smaller HVC of female zebra finches.

As stated above, the lack of FoxP2 sexual dimorphism reinforces our interpretation that dimorphic FoxP1 expression is not simply an epiphenomenon of cell density. Together, these findings suggest a role for FoxP1 as well as FoxP2 in the sexually dimorphic vocal learning of male zebra finches. We, thus, decided to investigate both FoxP1 and FoxP2 expression in humans.

**FOXP1 and FOXP2 expression overlaps in subcortical regions of human fetal brain**
Our data in the zebra finch, coupled with the articulation phenotype observed in affected members of the KE family, suggested to
us that FOXP1 and FOXP2 expression patterns would be conserved between songbirds and humans. Specifically, the human language phenotype that arises from a mutation in FOXP2, coupled with the overlapping expression of FoxP2 with FoxP1 in the striatum and thalamus of the zebra finch, hints at a combinatorial role for these genes in the development of vocal control circuitry. This hypothesis would be supported by a similar overlap in the developing human brain. Thus, in situ hybridization analysis was performed between 15 (data not shown) and 22 weeks gestation (Figs. 7, 8), when subcortical neurogenesis and migration are essentially complete and cortical neurogenesis is ongoing.

In the cortex, a complementary pattern of FOXP gene expression occurs in human embryos, with FOXP1 signals observed at more superficial layers than those of FOXP2 (Figs. 7C, E, 8A). Within the striatum, FOXP1 and FOXP2 are expressed in highly similar patterns, in the head and tail of nucleus caudatus and putamen (Figs. 7A–D, 8B), where the intensity of FOXP label is reminiscent of the strong FoxP signals within the songbird striatum. Strikingly, FOXP2 shows restricted expression within the GP, with high levels of expression in the GPi (Figs. 7B, C, 8B), which provides the principal source of output from the basal ganglia to the nucleus centrum medianum thalami (CM) and the major motor relay nuclei of the thalamus. As in the zebra finch, human FOXP1 and FOXP2 expression overlaps in the thalamus, with FOXP2 revealing more extensive expression, specifically in the CM and nucleus medialis dorsalis thalami, both regions with homologs in the avian DTZ (Veenman et al., 1997) thalami (Fig. 7C, D). More moderate signals arise from the nuclei anterior thalami, dorsal and ventral, and the nucleus parafascicularis thalami (Pf) (Fig. 7B, D). FOXP2 is expressed in the ventrobasal complex comprising the nucleus ventralis posterior lateralis/medialis (Fig. 7D). Similar to the VIA in the zebra finch (Medina et al., 1997), the ventral tier of the human thalamus exhibited strong FOXP2 expression, including nuclei ventralis anterior, lateralis, and posterior lateralis pars oralis (Fig. 7C, D). These nuclei have strong motor and premotor cortex connectivity, comprising key motor nuclei of the thalamus (Olszewski, 1952). Both genes also demonstrated significant expression in the nucleus subthalamicus bilaterally (Fig. 7C). Additionally, FOXP2 is strongly expressed in the nucleus ruber (Fig. 7D). The human brain regions of FOXP expression are key relays in essential motor control circuitry comprising premotor and posterior prefrontal pathways involved in motor planning and execution. This pattern of expression in specific subcortical structures for both FOXP1 and FOXP2 is entirely consistent with the putative role of these genes in pathways of sensorimotor integration that subserve vocalization and other complex learned motor movements. In no case did we observe asymmetry of FoxP gene expression.

**Discussion**

The discovery that FOXP2 is the monogenetic locus for a human language disorder affords the first opportunity to test a gene identified in the sole primate vocal learner, *Homo sapiens*, for its role in more experimentally accessible vocal learners, oscine songbirds. The corticostriatal abnormalities and speech disruption observed in humans bearing a FOXP2 mutation, coupled with the well-described corticostriatal song circuit in songbirds, suggested the exciting hypothesis that FoxP2 regulates common mechanisms for vocal learning. As a first test of this hypothesis, we identified the full-length homolog of FOXP2 in the zebra finch, an oscine songbird. The deduced amino acid FoxP2 sequence in the finch (Fig. 2) does not contain the two residues that, among primates, are specific to humans. However, it does possess five residues that differ from currently known mammalian forms. This finding sustains the possibility that among mammals and among birds, independent variation in FoxP2 secondary structure contributed to the capacity for vocal learning in certain species (Enard et al., 2002; Zhang et al., 2002; Clark et al., 2003).

Before the identification of the KE family mutation, research on Foxp2 focused on the lung airway epithelium, where Foxp1, Foxp2, and Foxp4 exhibit coordinate developmental expression...
with regions of distinction as well as overlap (Shu et al., 2001; Lu et al., 2002). Here, in the brains of an avian and a primate vocal learner, we find that FoxP1 and FoxP2 have both distinct and shared expression patterns in the cortex, striatum, and thalamus. Based on the human mutant phenotype, we predicted FoxP expression within the song circuit of the male zebra finch, a species in which only males learn to sing a courtship song. We find that FoxP2 is indeed expressed within area X of the striatum; however, this expression is not sexually dimorphic. In sharp contrast, FoxP1 exhibits several regions of sexual dimorphism. In two song nuclei, HVC and area X, FoxP1 expression overlaps with that of FoxP2 and is higher than in the surrounding regions. Expression within area X is of particular interest because this nucleus is the specific region of the striatum required for song development (Sohrabji et al., 1990; Scharff and Nottebohm, 1991) and that exhibits song-selective neuronal responses during singing (Jarvis and Nottebohm, 1997) or playback (Solis and Doupe, 2000) of the bird’s own song.

In regions of overlap, FoxP1 and FoxP2 could act as coregulators in the brain, as indicated in the lung (Shu et al., 2001) and by the capacity for Foxp1 to dimerize with other subfamily members (Wang et al., 2003; Li et al., 2004). In zebra finches, FoxP1 expression could confer a sexually dimorphic function on FoxP2 in sites where dimorphic FoxP1 expression overlaps with monomorphic FoxP2. The monomorphic expression in brain regions of song nuclei raises two possibilities. First, it could reflect the potential of females to learn to sing when given early hormonal treatment (Gurney and Konishi, 1980; Akutagawa and Konishi, 2001; Grisham et al., 2002) and, more generally, for females of other songbird species to sing. Simply put, a given FoxP molecule may be necessary, but not sufficient, for vocal learning and likely interacts with other proteins for that potential to be realized. In this regard, neural expression patterns of all FoxP subfamily members across avian phylogeny will be informative (Haesler et al., 2004). Second, monomorphic expression between male and female zebra finches in song control regions may highlight areas of sensory processing used by both sexes in perception of song (Brenowitz, 1991; MacDougall-Shackleton et al., 1998; Leitner and Catchpole, 2002).

Our results in the finch predicted that in humans, FOXP1, in addition to FOXP2, would be expressed in similar cortical, striatal, and thalamic patterns. The avian pallium bears homology to the mammalian cortex, however, a one-to-one correspondence between cortical and pallial layers is lacking (Reiner et al., 2004). Despite this structural difference, the cortical complementarity of human FOXP expression is reminiscent of that in the zebra finch pallium, where FoxP2 is diffusely expressed whereas robust FoxP1 expression is localized to the HD, mesopallium, and the song nucleus HVC. In line with our prediction, FoxP1 and FoxP2 expression overlaps in both songbird and human striatum, including within songbird area X and in the human nucleus caudatus and putamen. To the extent that mammalian and avian thalamic subregions have been compared (Medina et al., 1997; Veenman et al., 1997), FoxP gene expression is quite similar in human and songbird. This includes strong expression in motor structures within the songbird DTZ and in the vicinity of the VIA and the human IMMC and ventral tier. Sensory thalamus, in addition to motor thalamus, also expresses FoxP mRNA. Similarly, in mesencephalon, both sensory and motor structures are labeled in humans and songbirds. This expression pattern is compatible with a role for these molecules in movements that rely on sensory feedback (Konishi, 1965; Morrison and Nottebohm, 1993).

Interestingly, FoxP genes are expressed in regions of the zebra finch DTZ that are homologous to the CM and Pf in mammals (Veenman et al., 1997; Bruce et al., 2002). These thalamic nuclei are hypothesized to provide “attention-specific sensory information important for conditioned responses” in primates (Sidibe et al., 2002). In rats, Pf appears important for orofacial function (Tsumori et al., 2002, 2003). Yet, the Bas of the songbird, an orofacial control region implicated in feeding (Wild and Farabaugh, 1996), conspicuously lacks FoxP1 (Fig. 3A, B). This example again points to roles for FoxP molecules in learned, rather than purely innate, orofacial behaviors, a hypothesis that...
invites further study. One hint from the human behavioral data is that affected members of the KE family display no problem with musical pitch and intonation, yet are impaired on production and perception of rhythms (Alcock et al., 2000b). This central deficit in temporal patterning is consistent with a role for FoxP molecules in tasks that require sensory feedback to trigger or predict motor output.

The lack of asymmetry in human FOXP1 and FOXP2 neural expression is not surprising given the bilateral nature of the structural deficits observed in affected KE family members (Belton et al., 2003). In both humans and songbirds, FoxP expression occurs early in development, at a time when both hemispheres of the human brain are capable, albeit not equipotent, of giving rise to language later in development (MacWhinney et al., 2000; Vicari et al., 2000). Given the bilateral expression of the FoxP genes observed here, molecular understanding of the predominantly lateralized aspects of language function in humans remains as a significant challenge for future studies.

Our findings provide the first view of FOXP1 neural expression in humans. Furthermore, they complement and extend recent studies on FOXP2 expression in mammalian brain, because the human developmental stage examined here allows for finer localization to subcortical structures than in prior studies (Lai et al., 2003). Although we have focused on FOXP mRNA, findings in mouse (Ferland et al., 2003) and zebra finch (Haesler et al., 2004) brain indicate that mRNA and protein are localized similarly. The emergent pattern for FOXP2 is of robust cortical, basal ganglia, thalamic, and cerebellar expression. The complementary cortical expression of FOXP1 versus FOXP2 found in humans is similar to that in mouse (Ferland et al., 2003). An interesting difference is the discovery that human GPI expresses FOXP2 (Fig. 6C), because no such signal was detected in mouse (Ferland et al., 2003) and GPI was not previously reported on in human (Lai et al., 2003). This result provides additional support to a motor role for FOXP2, because GPI is the principal source of output from the basal ganglia to the major motor relay nuclei of the thalamus in humans.

Our findings, together with those of Haesler et al. (2004), provide the first picture of FoxP neural expression in another vocal learner, an oscine songbird, and do so across developmental time points. Persistent expression in adult zebra finches may indicate additional roles in the mature brain because other striatal transcription factors tend to exhibit developmental downregulation (Takahashi et al., 2003). Because the timeline and the specific structures for song learning are known, songbird research can further define the pathways for vocal learning that FoxP2 acts on and to discover new molecules that may be common to vocal learning (Clayton et al., 1988; Denisenko-Nehrbass et al., 2000; Akutagawa and Konishi, 2001), including FoxP1. In the zebra finch, the high levels of FoxP2 in striatum and thalamus, coupled with sexually dimorphic expression of FoxP1 in multiple song control regions including area X, may reflect combinatorial regulation by these proteins of the development of vocal control structures. Taken together, the similar patterns of FoxP gene expression in zebra finch and human suggest that FoxP1, in addition to FoxP2, is likely to play an important role in the formation and function of circuits for learned articulation requiring fine sequential motor control in songbird and human.

References


